Evaluation of the Aging Immune System
Using a Mouse Model of *Brucella* Infection

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Thesis submitted to the faculty of the
Virginia Polytechnic Institute and State University
in partial fulfillment of the requirements for the degree of

**Master of Science**
in
**Biomedical and Veterinary Sciences**

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**November 12, 2008**

**Blacksburg, Virginia, USA**

Keywords: Aging, *Brucella*, RB51-SOD, Murine models, Immunosenescence, Th17
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by

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**Abstract**

Aging is accompanied by dysregulated immune function resulting in increased susceptibility of the elderly to diseases caused by microbial pathogens. There exists a multitude of data suggesting decreased resistance of the elderly to a variety of intracellular pathogens but there is no data relating the effect of aging on the immune response against *Brucella*. To elucidate the mechanism of immune dysregulation in old, old and young DBA/2 and BALB/c mice were infected with wild-type *B. abortus* strain 2308. The old and young mice were also vaccinated with vaccine *B. abortus* strain RB51 over-expressing Cu-Zn superoxide dismutase (SOD) and then challenged with *B. abortus* strain 2308 to determine the effect of vaccination in old vs. young mice. Specific IgG1 and IgG2a response to *Brucella* antigens were also evaluated to determine the effect of aging on Th-specificity of the immune response against *Brucella* infection. The immune response in aged vs. young mice was further assessed using RT-PCR and cytokine antibody array to determine the type of T-helper response. The experimental results indicate that all mice, regardless of age, survived infection ranging from doses of $2 \times 10^4$ to $2 \times 10^8$ CFU. Though the older DBA/2 mice had a higher organism burden after 1 week of infection, these mice cleared *Brucella* infection more efficiently (5 weeks post-infection) than young mice. Vaccination with strain RB51 over-expressing SOD provided significant protection in
young DBA/2, young BALB/c and old BALB/c mice but not in old DBA/2 mice after strain 2308 challenge. The results also suggest that old mice produced a different magnitude of IgG1 and IgG2a response to bacterioferritin and SOD of *Brucella*. The data suggests that both Th17 as well as Th1 responses were accentuated in old mice as compared to young mice following infection with *Brucella*. How the Th17 and Th1 branches of adaptive immune system work together enabling old mice to clear *Brucella* better than young mice warrants future investigation.
Dedication

I would like to dedicate this work to my beloved father, Mr. Krishna Prasad (Malda, India) who is in heavenly abode now but his blessings will always be with me for my entire life. I love you, Papa!
Acknowledgement

I take this opportunity to express my deep sense of gratitude and reverence to my advisor, Dr. N. Sriranganathan, Professor, Department of Biomedical Sciences and Pathobiology, Virginia Tech for his precious guidance and critical counsel. He has played a very significant role in my life apart from my studies.

I express my gratitude to Dr. Stephen Boyle, Professor, Department of Biomedical Sciences and Pathobiology, Virginia Tech for providing his timely help and advice whenever I needed.

I wish to express my sincere gratitude to Dr. Kevin High, Professor, Wake Forest University School of Medicine, Winston-Salem, North Carolina and Dr. Ann Stevens, Associate Professor, Department of Biological Sciences, Virginia Tech as members of advisory committee for their co-operation in the pursuit of this study.

I express my gratitude towards Dr. Gerhardt Schurig, Dean, Virginia-Maryland Regional College of Veterinary Medicine, Virginia Tech for supporting me in my study through his timely advice.

I have a bouquet of special feelings and thanks for Dr. Abey Bandara, Assistant Professor (Research) for his valuable guidance and constant encouragement during the entire period of my research work.

Finally, I pay my regards to my parents and my brother, Suman for their continuous support and overwhelming love that has already keep me tuned. There is nothing great in world for me against their efforts and love.

Financial support from NIAID grant # AI057952 from National Institute of Health (NIH) is greatly acknowledged.
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**Abbreviations**

SOD - Cu-Zn Superoxide dismutase

BFR - Bacterioferritin

CFU - Colony Forming Unit

IgG - Immunoglobulin G

SDS - PAGE - Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

Th1 - T-helper 1 cell

Th2 - T-helper 2 cell

Th17 - T-helper 17 cell

RT-PCR – Reverse Transcription Polymerase Chain Reaction

NK Cells – Natural Killer Cells

ER – Endoplasmic Reticulum

MHC - Major Histocompatibility Complex

APC – Antigen Presenting Cell

LPS – Lipopolysachharide

TLR – Toll-like Receptors

DC – Dendritic Cell

IL-17 – Interleukin-17

IFN-γ – Interferon-gamma

OD – Optical Density

ELISA – Enzyme-linked Immunosorbent Assay

PBS – Phosphate Buffered Saline

SEM – Standard Error of the Mean

kDa - KiloDalton
CHAPTER 1

INTRODUCTION

One of the most remarkable occurrences observed in the history of human civilization is the continuing increase in the life expectancy. The reasons for this increase are advances in the technology and improved medical knowledge and practices. Life expectancy has been increasing in most parts of the world in past few centuries. The life expectancy of the human population in the 18th century was in the range of 20 to 35 years, which increased in the 21st century to an astonishing 80 years in world's developed countries (United Nations, 2002). Industrialized countries are now facing a sudden surge in the population of elderly people. For example, in United States of America, the percentage of persons aged 65 years and older relative to the entire population, increased from 4% in 1900 to 12% in 2006 and it is predicted that by the year 2030, one-fifth of the population will fall in this age-group. It has been a major challenge for the field of medicine to provide efficient medical care to the growing number of elderly. These older persons are at increased risk of developing infections due to a variety of pathogens. This has been attributed to a progressive, age-related, unidirectional decline in the immune responses in the aged people, a phenomenon known as immunosenescence. The increased susceptibility of the elderly to the infections due to intracellular pathogens like *Mycobacterium tuberculosis*, *Salmonella*, Influenza virus results in increased mortality and morbidity. Infectious diseases like influenza and pneumonia are some of the leading causes of death in the elderly population. Mice and humans generally exhibit a shift from Th1-predominance to a Th2-predominant response as they age. However, there is little data relating to effect of aging on immune response against *Brucella* infection.
Brucellosis is the widespread zoonotic infection worldwide with more than half a million new cases of infection annually. A renewed scientific interest in *Brucella* has been generated owing to classification of this organism as a class B bioterrorism agent (Greenfield, 2002). As few as 10-100 *Brucellae* are sufficient to cause infection through inhalation. Since no vaccine exists for human brucellosis, the disease poses a significant threat to the burgeoning population of elderly persons. The present study, therefore, has been designed to study the effect of aging on immune responses against an important zoonotic pathogen *Brucella* using a mouse model. There exists a variety of models in biogerontological research such as yeast, nematodes, fruit flies, hamsters, mice, rats and non-human primates. The use of mouse model in these studies has some advantages over other models in spite of the fact that mouse and human immune system have significant differences in terms of development, activation and their response to infection (Mestas, 2004). The mouse model is preferred owing to the rodent's short life-span, low cost and availability of a vast resource of reagents and knowledge regarding their biology and behavior. The present study has been designed to achieve the following objectives:

I. Determine the outcome of *Brucella* infection in old vs. young adult mice.

II. Determine the effect of strain RB51 vaccination on clearance of *Brucella* in old vs. young adult mice.

III. Determine the effect of aging on Th-specificity of the immune response to *Brucella* infection.

IV. Characterize the polarity of immune response (Th1, Th2 or Th17) in old vs. young adult mice to *Brucella* infection.
CHAPTER 2

REVIEW OF LITERATURE

Brucella: Introduction

*Brucellae* are small, Gram-negative, non-spore forming, facultative intracellular bacteria that cause brucellosis in a range of vertebrates. Brucellosis is an ancient disease and its occurrence dates back to the 79 AD, when tremendous volcanic eruptions of Mount Vesuvius completely buried two famous cities in Italy-Pompeii and Herculaneum. The anthropological examination of the skeletons recovered from these sites revealed bone lesions typical of brucellosis. This high incidence of brucellosis among the inhabitants of Roman Empire is attributed to the consumption of milk and milk products derived from sheep and goat (Capasso, 2002). The scanning electron microscopy analysis of the carbonized cheese found from Herculaneum also revealed the presence of coccobacilli-like bacteria morphologically resembling *Brucella*. In the 19th century, Sir David Bruce isolated this bacterium that was responsible for Maltese fever among the British soldiers stationed at Malta (Bruce, 1887). Dr. Bruce isolated the bacterium from human spleens and designated it as *Micrococcus melitensis*. The bacterium has been renamed as *Brucella melitensis* in the honor of Dr Bruce. In 1905, Zammit, a Maltese physician working with Mediterranean fever commission, isolated the organism from goat's milk and demonstrated the zoonotic nature of the organism (Zammit, 1905). Bang, a bacteriologist, in Denmark in the same period isolated the Gram-negative rods from aborted cattle and named the organism *Bacillus abortus*. In 1914, Traum isolated the third member of the group from an aborted pig fetus and named it as *B. suis*. Buddle
and Boyce discovered *B. ovis* as a cause of epididymitis in sheep and Carmichael and Bruner in 1968 isolated *B. canis* from aborted canine fetuses. *Brucella* was first reported to be recovered from sea mammals when the organisms were isolated from seals, porpoises and a dolphin in Scotland (Ross et al, 1994) and a bottlenose dolphin in USA (Ewalt et al, 1994). Brucellosis is the commonest zoonotic disease worldwide in the animal and human populations. Human brucellosis has a wide spectrum of clinical manifestations, ranging from fever to arthralgia, myalgia, fatigue and malodorous perspiration (Pappas et al, 2006). The invasion by *Brucella* of the reticulo-endothelial system leads to splenomegaly and lymphadenopathy. Brucellosis is transmitted to humans from infected domestic animals. Dairy products such as cheese, ice-cream made from unpasteurized milk serves as an important source of infection in general populations. *B. melitensis* is the most important zoonotic agent followed by *B. abortus* and *B. suis*. The main pathogenic species for domestic animals is *B. abortus* causing bovine brucellosis. *B. melitensis* is responsible for small ruminant brucellosis, *B. suis* for swine brucellosis, *B. ovis* for ram epididymitis and *B. canis* for canine brucellosis. Manifestation of the disease may range from abortion in pregnant animals and to orchitis or epididymitis in bulls. The genus *Brucella* consists of eight species depending on antigenic variation and primary host. *B. melitensis* (sheep and goat), *B. suis* (pigs), *B. abortus* (cattle), *B. ovis* (sheep), *B. canis* (dogs), *B. neotomae* (wood rats), *B. ceti* and *B. pinnipediae* (marine mammals). *Brucella* are phylogenetically related to plant pathogens and symbionts such as *Rhizobium* and *Agrobacterium*, intracellular animal parasites such as *Bartonella* and *Rickettsia* and to free living bacteria like *Ochrobactrum* and *Caulobacter* (Velasco et al, 1998).
Brucellae are facultative, intracellular pathogens that invade the professional and non-professional phagocytes and replicate within endosomal compartments associated with endoplasmic reticulum of macrophages (Moreno and Moriyon, 2002). Professional phagocytes are the main cells containing the pathogen in acute phase of infection. The main characteristic feature of the brucellae that distinguishes them from most of the other pathogenic microbes is that they do not exhibit classical virulence factors such as capsules, fimbriae, exotoxins, exoproteases, cytolysins, plasmids or lysogenic phages (Letesson et al., 2002). *Brucella* has a remarkable capacity to resist killing by professional phagocytes such as neutrophils and macrophages. The pathogen inhibits the degranulation of the stored granules in the neutrophils to evade the myeloperoxide-\( \text{H}_2\text{O}_2 \)-halide system. *Brucella* evades macrophage killing by preventing phagolysosomal fusion through rapid acidification of the phagosome following uptake (Porte et al, 1999). *Brucella* produces copious amount of \( \beta \)-glucan molecules upon infection which disrupt the macrophage membrane lipid rafts so that it can no longer serve as a signaling platform and as a result, *Brucella*-containing vacuoles (BCV) cannot fuse with lysosomes and permit *Brucella* replication (Arellano-Reynoso et al, 2005). But, the final stage in phagosomal maturation is formation of replication-permissive organelle derived from endoplasmic reticulum (ER). The fusion of the BCV with ER depends on expression of *Brucella* type IV secretion system, VirB (Boschiroli et al, 2002).

**Brucella: Immunity**

Immunity against *Brucella* involves antigen-specific T-cell (CD4+ and CD8+) and B cell (antibody) response. Different arms of the immune system including T
cells, B cells, Natural Killer (NK) cells and antigen presenting cells (APC) work synergistically to limit *Brucella* infection. APC consist of macrophages and dendritic cells, which along with NK cells constitute the first cellular barrier to infection by *Brucella*. The bacterial cell envelope, particularly the O-side chain of lipopolysaccharide (LPS) of *Brucella* is recognized by toll-like receptors present on the APCs (Huang et al, 2001). This result in internalization of bacteria through lipid rafts which lead to release of cytokines such as TNF-α, IL-6 and IL-12, which further activate the other innate immune cells. *Brucella* antigens are processed and presented along with MHC class I and II molecules to T-cell subsets and result in polarization of immune response after interaction with *Brucella*. Neutrophils and NK cells are part of the innate immune system playing an important role in *Brucella* immunity. Neutrophils are the first cells to encounter *Brucella* and utilize the ROS (reactive oxygen species) to kill most of the bacteria (Riley et al, 1984). NK cells are capable of killing microbe-infected cells and lysing Gram-negative bacteria (Garcia-Penarrubia et al, 1989). However, removal of NK cells has little influence on ability of mice to fight the *Brucella* infection indicating the role of other immune cells in *Brucella* immunity (Fernandes et al, 1995).

The macrophage and dendritic cells release the pro-inflammatory cytokines such as IL-12 and TNF-α and anti-inflammatory cytokines like IL-10 that act as a negative regulator of *Brucella*-specific immunity (Golding et al, 2001). The ability of *Brucella* to induce IL-12 production from APC enables it to drive the differentiation of Th0 cells to Th1 effector and memory cells. TNF-α is also necessary for macrophages to kill *Brucella*, which along with IFN-γ activate macrophages for anti-
*Brucella* activities. The Th1 cytokine, IFN-γ is also responsible for isotype switching of IgG to IgG2a isotype that is associated with complement fixation and protection against *Brucella* (Baldwin et al, 2006). In addition to CD4+, CD8+ T cells mediated cytotoxicity play an important role in *Brucella* immunity. Depleting CD8+ T cells with CD8 monoclonal antibodies resulted in significant increase in spleen CFU in strain 2308-infected BALB/c mice compared to undepleted controls (Yingst et al, 2003). γδ T cells are important cells bridging innate and adaptive immunity. One study showed that percentage of γδ T cells with Vγ9Vδ2 phenotype is increased in acute brucellosis and this subtype is activated by a small non-peptidic molecule present in *Brucella* and produce IFN-γ (Ottones et al, 2000). *Brucella* also stimulates the production of antibodies in a T cell-independent manner due to the presence of O-polysaccharide in its LPS from smooth strains. LPS activates B cells to produce antibodies known to play a role in opsonization and their ability to activate complement to kill bacteria (Betts et al, 1993).

**Immunosenesecence**

Immunosenescence is defined as the state of dysregulated immune function resulting in increased susceptibility of elderly to infectious diseases, cancer and autoimmune diseases (Pawalec, 1999). The aged immune system is not capable of mounting an effective immune response to infections and results in increased mortality and morbidity (Solana et al, 2006). The proportion of population of persons aged more than 65 years is expected to increase from 12.4% in 2006 to 19.6% in 2030 in the USA. Similarly, the number of persons aged more than 80 years is expected to increase from 9.3 million in 2000 to 19.5 million in 2030 (U.S. Census Bureau,
The developing countries will also experience the doubling in aged population in less than 30 years. Infectious diseases like pneumonia and influenza are among the leading causes of death in the persons aged 65 years and more. So, there is an increasing demand for research on infectious disease affecting the aged population (High, 2004).

Toll-like receptors (TLR) are the pattern recognition receptors, which recognize conserved molecular patterns on microbes known as pathogen associated molecular patterns (PAMPs). There are reports that suggest a decline in TLR function and expression due to aging that can result in poor adaptive immune response (Renshaw et al, 2002). The main cellular components of innate immunity are neutrophils, macrophages, dendritic cells and NK cells. Several lines of evidence suggest that NK-cell cytotoxicity is well preserved in healthy elderly individuals, but the cytokine production is decreased in the aged. There is a decline in phagocytic capacity and reduced superoxide production by macrophages in the elderly. The antigen presentation by macrophages is severely impaired in aged subjects due to diminished expression of MHC class II molecules both in humans and mice (Plowden et al, 2004). Any defect in antigen presenting ability of these innate immune cells will result in an impaired adaptive immunity. In contrast to macrophages, DCs retain the ability to present antigen to T cells; however DCs from elderly show diminished expression of IL-12 and costimulatory molecules (Uyemura et al, 2002).

Aging is associated with a continuous senescence of the immune system. It affects both innate and adaptive immunity, but there is severe deterioration of adaptive immunity with age. The main phenotype is the thymus involution with
age that results in a decrease in number of naïve T cells in the elderly, whereas there
is an increase in the effector-memory CD8+ T cells (Weng, 2006). In addition, there
is a decrease in the absolute number of CD4 and CD8 T cells. At the same time, there
is an increased propensity for skewing towards Th2 immune response with aging that
result in increased IL-10 production (Castle, 2000) along with decreased Th1
response. Humoral immunity is also affected by aging process as there is impairment
in primary and secondary responses to vaccination in the elderly with diminished
production of specific antibodies due to reduction in clonal expansion (Burns EA,
1997). There is marked decrease in the diversity of the antigen-recognition repertoire
with age (Goronzy and Weyand, 2005). While the effect of aging on adaptive
immunity is a well-documented phenomenon; the impact of aging on innate immunity
mostly remain unresolved.

Fig. 1: Population of persons 65 years or over in 2000 and 2030
**Aging and Infectious Disease**

A large collection of data exists on age-related decline in resistance to infection caused by intracellular pathogens. *Listeria* and *Toxoplasma* models in old mice showed a decrease in specific pathogen immunity with increasing age (Gardner and Remington, 1977). Similarly, impaired production of T cells is associated with decreased resistance of senescent mice to *Listeria* (Patel, 1981). An age-related inability to generate protective T lymphocytes in response to infection is found to be responsible for increased susceptibility of old mice to *Mycobacterium* (Orme, 1987).

There are some exceptions to the concept of increased susceptibility to infections in the aged. In a *Leishmania* model, senescent BALB/c mice developed resistance to infection due to spontaneous release of IL-12 by macrophages (Ehrchen et al, 2004). Similarly, senescent mice displayed an early resistance to mycobacterial infection in spite of impaired CD4+ T cell response. The growth of *Mycobacterium* within the lungs of old mice was decreased during first 3 weeks of infection. The transient resistance was due to increase in CD8+ T cells capable of producing IFN-γ in antigen-independent fashion (Vesosky et al, 2006). These senescent mice possess a mechanism that is responsible for the increased resistance to these pathogens. Characterization of this mechanism will provide a better insight into protecting the aged population from infectious disease like brucellosis. There are some methods of immunological restoration in the elderly including calorie restriction, exercise, Vitamin E supplementation and low dose cyclophosphamide treatment (Hirokawa and Utsuyama, 2002). In order to improve the response of elderly to vaccination, use of CpG oligodeoxynucleotides as an adjuvant in aged BALB/c mice (Maletto et al,
or induction of inflammatory cytokines via adjuvant (Haynes et al., 2004) looks promising in the elderly. According to a recent report, KGF (Keratinocyte growth factor) therapy may be useful for treating age-related thymopoietic insufficiency to restore the immune function in the elderly (Min et al., 2007).

**Th17 cell: An overview**

The directed development of CD4+ effector cells in response to cytokines secreted by cells of the innate immune system is a hallmark of the adaptive immune system. The CD4 response has been limited to the Th1-Th2 paradigm where Th1 cells develop in response to sequential action of IL-12 and IFN-γ to combat infections due to intracellular pathogens; in contrast Th2 cells develop in response to IL-4 and help in antibody production. The transcription factors T-bet, STAT1 and STAT4 are important for the development of Th1 cells, while GATA-3 and STAT6 support Th2 development. Recently, a new T helper cell subset has emerged that plays an important role not only in host defense, but also in autoimmune diseases. The research on this new subset of T cells has spurred in the last few years. IL-17-producing CD4+ T cells develop by a pathway distinct from Th1 and Th2 lineage development (Harrington et al., 2005). These cells are designated as Th17 (Fig. 2) and are linked to the expression of a variety of cytokines. Th17 cells were initially believed to make only IL-17A but now found to secrete other cytokines including another member of the IL-17 family, IL-17F and IL-22, a member of the IL-10 family (Stockinger, 2007).
IL-17A is the founding member of the IL-17 cytokine family. There are six members in this family; IL-17A through F. IL-17A and IL-17F are homologous in their amino acid sequences and genes encoding these cytokines are present on the same chromosome in mice and humans. IL-17 expression was first detected in many inflammatory diseases like rheumatoid arthritis, systemic lupus erythematosus (SLE). IL-17A and IL-17F were first thought to be the product of CD4+ T cells, but it is now evident that CD8+ T cells, γδ T cells and neutrophils also produce these cytokines (Weaver et al, 2007). IL-23 was reported to be an important cytokine for the development of Th17 cells but later it was found that TGF-β and IL-6 are the cytokines responsible for the generation of Th17 cells from naïve T cells (Veldhoen et al, 2006). Further research confirmed the reciprocal development pathway for Th17 and T regulatory cells where TGF-β in the absence of IL-6 support the generation of regulatory T cells (Bettelli et al, 2006). Th17 cells do not express T-bet or GATA-3, transcription factor for Th1 and Th2 cells. Recently, ROR-γt, a retinoic acid-related
orphan receptor, has been implicated as a transcription factor for Th17 commitment and differentiation (Ivanov et al, 2006). Although IL-23 is not involved in the generation of Th17 cells but plays an important role in maintaining the effector function of Th17 cells (Mangan et al, 2006). IL-23 is a member of IL-12 cytokine family with p40 subunit in common with IL-12 and a unique p19 subunit. IL-23 signals through a heterodimeric complex composed of IL-23R and IL-12Rβ1 whereas IL-12R consists of IL-12Rβ1 and IL-12Rβ2 subunits. IL-12Rβ1 is expressed constitutively while IL-12Rβ2 and IL-23R is induced upon T cell activation. IL-23R expressed on Th17 cells makes them responsive to IL-23 which induces the clonal expansion of Th17 cells (Bettelli et al, 2007). IL-27, another cytokine of IL-12 family, has been implicated in the negative regulation of Th17 cell development (Stumhofer et al, 2006).

**IL-17 and host defense**

IL-17 is not only involved in autoimmune diseases, it also participates in host defense. The first report of IL-17 in host defense was reported in a *Klebsiella pneumoniae* infection, where IL-17 was released from CD4+ T cells in the lung. IL-17 resulted in augmented neutrophil recruitment and enhanced bacterial clearance after challenge with *Klebsiella* (Ye et al, 2001). IL-17 was therefore placed at the interface of adaptive and innate immunity by mediating the mobilization of neutrophils. *Bordatella pertussis* whole-cell vaccines activate innate immune cells through TLR4 and direct the induction of Th17 cells which in turn mediates protective immunity to *Bordatella pertussis* (Higgins et al, 2006). Similarly the protective role of IL-23/IL-17 axis has been documented in the case of *Mycoplasma*.
*pneumoniae* infection, where IL-17 production is IL-23 dependent and contributes to host defense through neutrophil recruitment (Wu et al, 2006). IL-17 also increases components of innate immunity such as β-defensin-2 and MIP-3 through which it also contributes to the airway innate mechanism (Kao et al, 2004). Recently IL-23/IL-17 axis has been found to play an important role in *M. tuberculosis* infection, where it establishes a protective CD4+ T cell response after vaccination and during challenge (Khader et al, 2007).

**IL-17 and aging**

There are only three reports that link IL-17 with aging. According to the first report, aging induces a shift in the vascular cytokine expression toward a proinflammatory profile and expression of IL-17 is significantly increased with aging in mice (Csiszar et al, 2003). The second report is related to virally induced pneumonia in mice, where there is a diminished production of IL-17 and other inflammatory mediators including IL-6, Keratinocyte chemoattractant (KC) in aged mice (Bonville et al, 2007). IL-17 has been found to be upregulated in aged mice due to *Brucella* infection in comparison to young mice and it is suggested that IL-17 could play an important role in the immune system of older mice enabling them to clear *Brucella* infection more effectively than the young adult mice (High, 2007). Recently, it has been reported that splenic CD4+ T cells from old mice produce significantly higher amount of IL-17 and another Th17 cytokine, IL-6 than young mice when incubated with T cell receptor antibodies (Huang et al, 2008).
CHAPTER 3

MATERIALS AND METHODS

1) Outcome of Brucella infection in aged and young adult DBA/2 mice

Animals and Bacteria- Young adult (2-3 months old) and old (16-18 months old) female DBA/2 mice were purchased from the rodent colony of the National Institute of Aging (Harlan-Tekland; Madison, WI).

Infection with B. abortus – a) In the first study, old and young DBA/2 mice (n = 20) were infected with $2 \times 10^4$ CFU of B. abortus strain 2308 and 5 mice from each age group (old and young) were killed on week 1, week 3, week 5 and week 8 to determine the CFU/spleen. b) In the second study, mice were inoculated intraperitoneally with different dosage of B. abortus strain 2308 i.e. $2 \times 10^4$, $2 \times 10^6$ and $2 \times 10^9$ CFU. Uninfected, control mice were injected with saline alone (100µl). These mice (n = 9-10) were killed by putting in a CO$_2$ chamber on day 35 and CFU/spleen was determined as previously described (He et al, 2002).

2) Protection from strain 2308 infection by strain RB51-SOD immunization

Animals and Bacteria- Young adult (2-3 month old) and old (16-18 month old) female DBA/2 and BALB/c mice were purchased from the rodent colony of the National Institute of Aging (Harlan-Tekland; Madison, WI). B. abortus strain 2308 was used to infect the mice and strain RB51 overexpressing SOD was used to immunize the mice.
Immunization with RB51-SOD - Mice were inoculated intra-peritoneally with $3.4 \times 10^8$ CFU of *B. abortus* strain RB51-SOD and other groups of mice were injected with saline alone. These mice were allowed to recover for 5 weeks.

Challenge with *B. abortus* strain 2308 - At the end of 5 weeks, all the mice (vaccinated and unvaccinated) were challenged with $2 \times 10^4$ CFU of *B. abortus* 2308. After 2 weeks, these mice were killed and CFU/spleen was determined according to the method described above.

3) **Effect of aging on Th-specificity of the immune response to Brucella infection.**

Materials, bacterial strains, plasmids and medium - Restriction endonucleases, *BamHI* and *HindIII*, and T4 DNA ligase were purchased from Promega Corporation (Madison, WI). PCR Supermix was obtained from Sigma Aldrich Inc. (St. Louis, MO). pRSETa vector (Invitrogen, CA) was used to clone and express the two genes, Bacterioferritin (*bfr*) and Cu-Zn Superoxide dismutase (*sodC*). *Escherichia coli* DH5α was used as a host in standard cloning experiments and was grown in Luria Bertani (LB) medium. *E. coli* BL21 cells were used for *bfr* and *sodC* expression and the strain bearing plasmid pRSET were grown in LB supplemented with 50 µg/ml ampicillin *B. abortus* genomic DNA was available from our laboratory.

A) **Cloning of bfr and sodC gene**

Based on the sequence of *bfr* gene, two primers- Forward containing *BamHI* restriction site (5'- **CCC**GGATCCATGAAAGGCGAACC**AAA**AGGTCATC**-3') and Reverse with *HindIII* restriction site (5'- **CCC**AAGCTTTACTCAGCTTCGC**GG**GGCGCC**-3') were designed to amplify
by PCR. The PCR amplification protocol consisted of a denaturation at 94°C for 5
min followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s,
extension at 72°C for 1 min, and a final hold for an extra 10 min at 72°C. Similarly,
two primers – Forward containing BamHI restriction site (5’-CCC<sub>GGATCC</sub>CATGAAGTCTATTATTTATTGCATCG-3’) and Reverse with HindIII
restriction site (5’-CCC<sub>AAGCTT</sub>TTATTCGATCACGCAGCCGCAAA-3’) were
designed for sodC gene. PCR conditions were same as used for bfr. The amplified
PCR fragment were directionally cloned into pRSETa vector according to
manufacturer's protocol and then transformed into E. coli DH5α. The cloning vector
was extracted from the cell culture using a QIAprep Spin Miniprep kit (Qiagen,CA).
The cloning of these two genes were confirmed by restriction digestion of the
extracted plasmids containing two genes with the enzymes, BamHI and HindIII (these
restriction sites were designed in the forward and reverse primers respectively) and
separation of fragments on an agarose gel.

B) Expression of sodC and bfr gene

Plasmids with either sodC or bfr were transformed into E. coli BL21 cells
following the manufacturer's protocol. 5 ml. of an overnight culture of the
recombinant clone was inoculated into 500 ml of LB broth containing 50 µg/ml of
ampicillin and grown at 37°C with vigorous shaking. When the OD of the culture
reached 60 Klett units, the culture was induced with IPTG to a final concentration of
1mM. After 2 hours of induction, the culture was washed thrice with PBS and
resuspended in 8 ml of lysis buffer and incubated for 30 minutes with shaking.
Proteins from the lysed cells were separated by 15% SDS-PAGE and stained with Coomassie Brilliant Blue (Sambrook et al, 2001).

C) Purification of recombinant BFR and SOD protein

Polyhistidine-tagged fusion protein was purified under denaturation conditions by metal chelate affinity chromatography (Qiagen, USA), with certain modifications introduced in the purification parameter. Induced cells from 500 ml culture were resuspended in 8 ml of lysis buffer. It was held at room temperature for 1 hour with intermittent mixing. The cell debris was removed by centrifugation at 12000 x g (Tomy TX 160, CA) for 20 min. The supernatant was mixed with 2 ml of Ni-NTA resin and left for 1 hour with intermittent mixing. The mixture is then packed into a 5 ml Polypropylene column (Qiagen, CA) and washed thoroughly with Buffer C. The fusion protein was then eluted with Elution buffer E for successive 15 times with 1 ml volume and all the fractions were checked by SDS-PAGE for the presence of the desired protein. The fractions with the desired protein were pooled together and was dialyzed using a dialysis cassette (Pierce, USA) and dialyzed against PBS for 72-96 hours. Concentration of recombinant protein after dialysis was determined by using BCA protein assay kit (Pierce, USA).

Western Blotting analysis

The eluted protein samples and cell lysate were separated by SDS-PAGE and then transferred to nitrocellulose membrane. The entire procedure was performed according to the protocols of QIAexpress system (Qiagen, USA), which is based on using anti-histidine tag antibodies. The polyhistidine tag is already present in the recombinant proteins.
D) ELISA

Total IgG, IgG1 and IgG2a titers vs. two Brucella proteins, bacterioferritin (BFR) and Cu\(^{++}\)/Zn\(^{++}\) Superoxide dismutase (SOD), were assessed by ELISA as follows. Recombinant SOD and BFR were purified from E. coli transformed with the B. abortus bfr or sodC gene in pRSET vector. Flat-bottomed 96-well polystyrene microtiterplates (Nunc, Denmark) were coated with 100 µl of SOD or BFR (20 µg/ml) suspended in 0.05 mM sodium bicarbonate buffer (pH 9.6). Purified mouse IgG (Sigma Aldrich Inc., St. Louis, MO), mouse IgG1 (Sigma Aldrich Inc., St. Louis, MO) and mouse IgG2a (Sigma Aldrich Inc., St. Louis, MO) were used to coat the 96-well plate starting from 625 ng/well to 312 pg/well for generation of standard curve. Each plate was incubated at 4°C overnight. Plates were washed three times with wash solution (PBST: PBS (pH 7.4) with 0.05% (v/v) Tween 20). Then plates were incubated with 100 µl of blocking solution of 1% (w/v) bovine serum albumin (Fisher scientific, Fair Lawn, NJ) for 1 h at room temperature and washed three times with PBST. Each sample of sera was diluted 1:20 in blocking solution and 50 µl of the diluted serum added to six wells of a 96-well plate. The plates were incubated at room temperature for 2 h. After three washes with PBST, goat anti-mouse IgG (Sigma Aldrich Inc., St. Louis, MO), goat anti-mouse IgG1 (Sigma Aldrich Inc., St. Louis, MO) and goat anti-mouse IgG2a (Sigma Aldrich Inc., St. Louis, MO) were added at 1:2500 dilution in duplicate. After three washes with PBST, each well was incubated with 100 µl of 1:1000 dilution of anti-goat whole IgG rabbit antibodies conjugated to horseradish peroxidase (ICN Biomedicals, Aurora, OH) diluted in blocking solution for 1 h at room temperature. After five washes with PBST, 100 µl of TMB [(3, 3’, 5,
5’-tetramethylbenzidine) peroxidase substrate (KPL, Gaithersburg, MD)] was added per well and plate was incubated for 20 min in the dark at room temperature. The colorimetric reaction was stopped by adding 50 µl of 2 N H$_2$SO$_4$ per well and optical density was measured at 450 nm using an ELISA plate reader.

4) **Determination of production of Th1, Th2 and Th17 cytokines and transcription factors during in vivo Brucella infection in old vs. young BALB/c mice.**

**Animals and Bacteria** - Young adult (2-3 month old) and old (16-18 month old) BALB/c mice were purchased from the National Institute of Health rodent colony (Harlan-Tekland; Madison, Wisconsin). *B. abortus* 2308 was from the culture collection of Dr. Sriranganathan's laboratory.

**Brucella abortus infection** – Mice were infected intra-peritoneally with 2 x 10$^4$ CFU of *B. abortus* strain 2308. Uninfected, control mice were injected with PBS alone. Mice were killed on day 0 (control) and day 1, day 3, day 7 and day 14 post-infection and liver from these mice were harvested. Subsequently, liver samples from these mice were flash-frozen in liquid nitrogen immediately and stored at -70°C. Similarly, blood was collected through retro-orbital bleeding using heparinized capillary tubes and serum was separated from the blood samples. Serum samples were stored at -70°C.
RT-PCR

Isolation of total RNA-Livers stored at -70°C were thawed and a portion weighing 30 mg were cut. Total RNA was isolated as per the manufacture's protocol (RNeasy, Qiagen) prescribed for tissues. RNA purity and concentration were determined by spectrophotometric absorbance at 260 and 280 nm using Nanodrop spectrophotometer (Dupont). Total RNA was subjected to DNase treatment according to the following protocol-

DNase treatment and removal of reagents( DNA-free Ambion)

10µg of total RNA was taken and subjected to DNase treatment in a total volume of 50 µl:

1. 5µl 10X DNase I Buffer.
2. 2.5 µl (2 U/µl) DNase 
3. 10 µg RNA sample
4. RNase-free water to a final volume of 50 µl.
5. Incubated at 37°C for 30 min.
6. 5 µl DNase inactivation reagent was added.
7. It was incubated for 2 min. at room temperature with occasional mixing.
8. Spun at 12,000 rpm for 1.5 min at room temperature, then supernatant was transferred to a new tube.
9. After DNase treatment, it is necessary to run a PCR for GAPDH gene to confirm DNA elimination and the concentration and purity of RNA was measured using a Nanodrop spectrophotometer (Dupont).
Reverse Transcription protocol (Promega)

1. 20 µl reaction was prepared by adding the following reagents in the order:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>MgCl₂</td>
<td>4 µl</td>
</tr>
<tr>
<td>Reverse Transcription buffer</td>
<td>2 µl</td>
</tr>
<tr>
<td>dNTP Mixture</td>
<td>2 µl</td>
</tr>
<tr>
<td>Inhibitor</td>
<td>0.5 µl</td>
</tr>
<tr>
<td>AMV Reverse transcriptase</td>
<td>0.75 µl</td>
</tr>
<tr>
<td>Oligo (dT) primer</td>
<td>1 µl</td>
</tr>
<tr>
<td>RNA sample</td>
<td>1 µg*</td>
</tr>
<tr>
<td>Nuclease Free water to a final volume of</td>
<td>X µl</td>
</tr>
<tr>
<td>Final volume</td>
<td>20 µl</td>
</tr>
</tbody>
</table>

* Note: DNA-free (Ambion) treated RNA should comprise only 20% of an RT-PCR reaction because inactivation of RT reaction.

2. The reaction mixture was incubated at 42°C for 60 min.

3. Sample was heated at 95°C for 5 min, then incubated at 0-5°C for 5 min. Store the first-strand cDNA at –80°C until use.

4. PCR amplification of the cDNA was performed with PCR Supermix (Sigma Aldrich Inc., St. Louis, MO) using the primers specific for different cytokines (Table 1).
Table 1: Oligonucleotide primers used in the RT-PCR for the amplification of each target gene and their product size.

<table>
<thead>
<tr>
<th>CYTOKINE</th>
<th>PRIMER SEQUENCE</th>
<th>PRODUCT SIZE (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-17A</td>
<td>Forward 5'-GCTCCAGAAGGCCCTCAGA-3' Reverse 5'-AGCTTTCCCTCCGCATTGA-3'</td>
<td>141</td>
</tr>
<tr>
<td>IL-17B</td>
<td>Forward 5'-TTACCATTTCATCTTCC-3' Reverse 5'-CCCTTTCTTGGTTTTTG-3'</td>
<td>210</td>
</tr>
<tr>
<td>IL-17C</td>
<td>Forward 5'-GATATCGCATCGACACAGAT-3' Reverse 5'-TGCTTCACGGCCTGTTTG-3'</td>
<td>101</td>
</tr>
<tr>
<td>IL-17D</td>
<td>Forward 5'-ATGTTGGGAGACTGTGCTCGGCTGCGCTTCC-3' Reverse 5'-GGACCTGATGCGAGGAAGCTGGGC-3'</td>
<td>498</td>
</tr>
<tr>
<td>IL-17E</td>
<td>Forward 5'-CGGGACAAGATGGAAGACTT-3' Reverse 5'-CAGCAACTCTGCTACAGCAAA-3'</td>
<td>92</td>
</tr>
<tr>
<td>IL-17F</td>
<td>Forward 5'-ATGGTCAAGTCTCTTGTGCTACTGTGATGTT-3' Reverse 5'-TCAGGCGCTTTGGTGGAAATGGC-3'</td>
<td>462</td>
</tr>
<tr>
<td>IL-23p19</td>
<td>Forward 5'-AATAAATGTGCCCCGTATCCA-3' Reverse 5'-CTGGAGGAGTTGGCTAGTC-3'</td>
<td>213</td>
</tr>
<tr>
<td>IL-6</td>
<td>Forward 5'-CAGAAATTGCGCATCGACACACCTTTCTTCA-3' Reverse 5'-CAGGATACCATCTACACAGAC-3'</td>
<td>140</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Forward 5'-TCAGGCGCTCTGACTCTGTGACG-3' Reverse 5'-GGTTCATGTCATGGATGGTC-3'</td>
<td>446</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Forward 5'-GCATCTTGGGCTTTGCAGCT-3' Reverse 5'-CGACTCTTTCTTCCGGCTTCT-3'</td>
<td>322</td>
</tr>
<tr>
<td>IL-12p35</td>
<td>Forward 5'-GAGGACTTGAAGATGTTACCC-3' Reverse 5'-TTCTATCTGTTGAGGTGGGC-3'</td>
<td>314</td>
</tr>
<tr>
<td>IL-12p40</td>
<td>Forward 5'-CGTGCTCATGCTGCTGGAGC-3' Reverse 5'-CCTCATGCTAAGGTTCCGGGC-3'</td>
<td>561</td>
</tr>
<tr>
<td>IL-12Rβ1</td>
<td>Forward 5'-GGCAACATTTGTTTCTGGTG-3' Reverse 5'-ATTCTTGGGGTTCTGGAGGC-3'</td>
<td>379</td>
</tr>
<tr>
<td>IL-12Rβ2</td>
<td>Forward 5'-GGAGGTACATGTTGGAAATGGA-3' Reverse 5'-GGCCTGCTATGCAATTTGC-3'</td>
<td>379</td>
</tr>
</tbody>
</table>
Table 1 continued……..

<table>
<thead>
<tr>
<th>CYTOKINE</th>
<th>PRIMER SEQUENCE</th>
<th>PRODUCT SIZE (bp)</th>
</tr>
</thead>
</table>
| IL-27p28      | Forward 5'-GGCCATGAGGCTGGATCTC-3'  
Reverse 5'-AACATTTGAAATCTGCAGGCA-3' | 75                |
| IL-15         | Forward 5'-GTGACTTTTCCACAGGGTGC-3'  
Reverse 5'-TCACATTCCTGTGAGCCAGA-3' | 180               |
| iNOS          | Forward 5'-CTTCCGAAGTTTCTGGCAGCAGCG-3'  
Reverse 5'-GAGCCTCTGTGCTTGGGCTCCTC-3' | 480               |
| CRAMP         | Forward 5'-GGATGAGAAATAATGAGGTGCA-3'  
Reverse 5'-TTTAGGAAATCCAGAAACAGGC-3' | 607               |
| IL-4          | Forward 5'-TCGTCATGACGACAGAGAG-3'  
Reverse 5'-CGAGTAATCCGATTGCCATGA-3' | 446               |
| FasL          | Forward 5'-CGGTGTTATTTTTCATGGTTTCTGG-3'  
Reverse 5'-CTTGTGTTTATTGGGGCTGTTG-3' | 380               |
| T-bet         | Forward 5'-AACCAGTATCTTTGTCCTCCGC-3'  
Reverse 5'-TGTCGCAAATCTGGGAAGGATAG-3' | 436               |
| GATA-3        | Forward 5'-GAAGGCATCCAGACCCGAAAC-3'  
Reverse 5'-ACCCATGGCGGGCTGACCATGC-3' | 255               |
| ROR-γt        | Forward 5'-CCGCTGAGAGGGCTTCAC-3'  
Reverse 5'-TGCAAGTAGGCACCTATTACA-3' | 230               |
| GAPDH         | Forward 5'-ACCACAGTCCATGACCATC-3'  
Reverse 5'-GTCCACACCCCTGCTGTA-3' | 200               |
| CXCL9         | Forward 5'-GATCAAACTGCCTAGATCC-3'  
Reverse 5'-GGCTGTGTAACAACCAGAG-3' | 399               |
| CXCL10        | Forward 5'-TGAGCAGAGATGTCTGGAATC-3'  
Reverse 5'-TCGCCACCTCCACACAGCCTACAG-3' | 384               |
| IL-27R        | Forward 5'-TGAAGCCAGACACACACCTC-3'  
Reverse 5'-CACACAGGCTTGGGTCTC-3' | 407               |
Cytokine antibody array

In addition to analysis of cytokine message by RT-PCR, cytokine antibody array (RayBiotech cytokine antibody array C series) was also performed according to the manufacturer's protocol to have a semi-quantitative estimation of different cytokines at the protein level. Serum sample collected from these mice were used in this experiment. Intensity of a given spot was normalized to the mean of positive control spots on each membrane. Intensity was calculated for each spot on the array for different cytokines and analysis was performed with the software provided by RayBiotech (Norcross, GA).

Statistical analyses

Differences between groups for continuous variables were determined by Student’s T-test or Wilcoxon Rank Sum test (using log-transformed values for CFU/spleen determination). For differences within each age group at different time points, ANOVA for repeated measures was used to determine significance. For all analyses, a level of p < 0.05 was considered significant.
CHAPTER 4

RESULTS

1) Clearance of *Brucella* infection in old vs. young mice

This experiment was performed to evaluate the effect of age on host defense against brucellosis. In the first study, one week after infection with strain 2308, the mean CFU/spleen in older mice was higher than that of young adult mice (p = 0.02). However, by week 3, older mice had equivalent CFU/spleen and by 5 or 8 weeks after infection, the CFU/spleen in older mice was 1–2 logs lower than the CFU in young adult mice (Fig. 3). In the second study; all mice, regardless of age, survived infection ranging from doses $2 \times 10^4 - 2 \times 10^8$. An infectious dose of $2 \times 10^9$ CFU of *Brucella* resulted in death of all 10 young mice in less than 72 hours, whereas 7 out of 9 old mice died in less than 72 hours, one died at one week and one survived until sacrifice 8 weeks later (Fig. 4). Old mice (n=9) infected with $2 \times 10^4$ and $2 \times 10^6$ CFU of *Brucella* also showed significantly lower burden of organisms per spleen in comparison to young infected mice (n=9) at 5 weeks post-infection (Fig. 5 and 6).
Fig. 3: Kinetics of Brucella clearance in old vs. young DBA/2 mice infected with 2 x 10^4 CFU of Brucella 2308.

Fig. 4: Survival curve in old vs. young DBA/2 mice infected with 2 x 10^9 CFU of Brucella 2308.
Fig. 5: Burden of organisms (mean ± SEM) in the spleen of young and old adult DBA/2 mice five weeks after infection with $2 \times 10^4$ CFU of strain 2308. (**p < 0.01)

Fig. 6: Burden of organisms (mean ± SEM) in the spleen of young and old adult DBA/2 mice five weeks after infection with $2 \times 10^6$ CFU of strain 2308 (**p < 0.01)
2) Protection from *Brucella abortus* 2308 infection by strain RB51-SOD immunization in old vs. young mice.

In this experiment, the ability of strain RB51-SOD vaccine in providing protection from strain 2308 infection was compared between old and young BALB/c and DBA/2 mice. The results have been divided into three subheadings:

A) **Effect of mouse strain** - The difference of CFU/spleen between unvaccinated mice of both the strains were significant in the same age group (i.e. old DBA/2 unvaccinated vs. old BALB/c unvaccinated and young DBA/2 unvaccinated vs. young BALB/c unvaccinated). (Fig. 7A). But the difference between the strains was not significant when the mice were vaccinated prior to challenge (Fig. 7B).

B) **Effect of vaccination** - It was found that young mice of both the strains were significantly protected from strain 2308 infection (Fig. 8A). Aged mice of BALB/c strain were protected significantly from strain 2308 infection by strain RB51-SOD vaccine, but this was not significant in case of old mice of DBA/2 strain (Fig. 8B).

C) **Effect of age** - The results suggest that the difference of CFU/spleen between old and young mice of the same strain was not significant whether they were vaccinated or not vaccinated (Fig. 9A and 9B).
Fig. 7A: Mean ± SEM CFU/spleen in old DBA/2 vs. BALB/c mice and young adult DBA/2 vs. BALB/c mice two weeks after challenge with strain 2308 in mice that were not immunized (control) (* p < 0.05).

Fig. 7B: Mean ± SEM CFU/spleen in old DBA/2 vs. BALB/c mice and young adult DBA/2 vs. BALB/c mice two weeks after challenge with strain 2308 in mice that were immunized with RB51-SOD five weeks prior to infection with strain 2308 (p > 0.05 for both the cases).
Fig. 8A: Mean ± SEM CFU/spleen in Young adult DBA/2 or BALB/c mice two weeks after challenge with strain 2308 in mice that were either naïve or immunized with $2 \times 10^8$ strain RB51-SOD five weeks prior to infection with strain 2308 (** p < 0.01).

Fig. 8B: Mean ± SEM CFU/spleen in old DBA/2 or BALB/c mice two weeks after challenge with strain 2308 in mice that were either naïve or immunized with $2 \times 10^8$ strain RB51-SOD five weeks prior to infection with strain 2308 (* p < 0.05).
Fig. 9A: Mean ± SEM CFU/spleen in old vs. young BALB/c mice two weeks after challenge with strain 2308 in mice that were either naïve mice or immunized with $2 \times 10^8$ strain RB51-SOD five weeks prior to infection with strain 2308 (p > 0.05 for both the cases).

Fig. 9B: Mean ± SEM CFU/spleen in old vs. young DBA/2 mice two weeks after challenge with strain 2308 in mice that were either naïve mice or immunized with $2 \times 10^8$ strain RB51-SOD five weeks prior to infection with strain 2308 (p > 0.05 for both the cases).
3) **Cloning, expression and purification of Bacterioferritin**

   a. Gene cloning- The bacterioferritin gene (*bfr*) was successfully amplified by PCR from the total *B. abortus* genomic DNA. The PCR product was 495 bp in length (Fig. 10). The amplified gene was run on 1% agarose gel and purified with a gel purification kit (Qiagen). It was cloned into pRSETa vector (Fig. 11) at the *BamH*I and *HindIII* restriction sites (designed into the forward and reverse primer respectively).

   b. For confirmation of correct genes, plasmids were extracted and subjected to restriction digestion with *BamH*I and *HindIII* overnight. Positive clones were confirmed by running the restriction digested products on a 1% agarose gel (Fig. 12).

   c. Expression and purification of recombinant protein – The expression vector of pRSET-BFR was transformed into *E. coli* BL21. According to the sequence of pRSET vector, the target fusion proteins also contain 6X histidine residues at the amino-terminal region. Following induction and harvesting, the lysed cells were mixed with Ni-NTA agarose. Then, it was subsequently eluted with elution buffer. These serially eluted fractions along with the cell lysate were analyzed by SDS-PAGE (Fig. 13). The molecular weight of the BFR recombinant protein was found to be approximately 22 kDa.

   d. Dialysis of the recombinant protein – All the eluted fractions were analysed by SDS-PAGE for the presence of the protein. Those fractions that contained BFR were then pooled together and dialysis was performed to remove the urea. The dialysed protein fraction was also subjected to SDS-PAGE analysis to confirm the presence of the recombinant protein (Fig. 15).
e. Western Blotting - The eluted fractions were subjected to Western blotting using His$_6$ tag anti-serum (Invitrogen) (Fig. 14).

4) **Cloning, expression and purification of superoxide dismutase**

   a. Gene cloning - The sodC gene was successfully amplified by PCR from the total *B. abortus* genomic DNA. The PCR product was 521 bp in length (Fig. 16). The amplified gene was separated electrophoretically on 1% agarose gel, purified and ligated into pRSETa vector at the *BamHI* and *HindIII* restriction sites.

   b. For confirmation of the plasmid with appropriate insert, plasmids were extracted by using plasmid Miniprep kit (Qiagen) and subjected to restriction digestion with *BamHI* and *HindIII* overnight. Positive clones were confirmed by running the restriction digested products on a 1% agarose gel (Fig. 17).

   c. Expression and purification of recombinant protein – The expression vector of pRSET-SOD was transformed into *E. coli* BL21 cells. Following induction and harvesting, the lysed cells were mixed with Ni-NTA agarose. Then, it was subsequently eluted with elution buffer. These serially eluted fractions were analyzed by SDS-PAGE (Fig. 18). The molecular weight of the Cu-Zn superoxide dismutase (SOD) recombinant protein was found to be around 18 kDa.

   d. Dialyses of the recombinant protein – All the eluted fractions were analyzed by SDS-PAGE for the presence of the protein. The positive fractions were then pooled together and dialysis was performed to remove the urea. Then, the dialyzed sample was analyzed by SDS-PAGE (Fig. 19).
e. Western Blotting - The eluted protein fractions were also subjected to Western blotting using His\textsubscript{6} tag anti-serum (Invitrogen) to confirm the presence of the recombinant SOD protein (Fig. 20).

5) **Determination of total IgG, IgG1 and IgG2a against Bacterioferritin**

   a. Total IgG - Old mice infected with *B. abortus* produced significantly higher levels of IgG compared to uninfected mice on week 3 and week 5 whereas young mice infected with *B. abortus* produced significantly higher levels of IgG at all time points in comparison to young uninfected mice (Fig. 21A) There was no significant difference between total IgG level of old and young mice infected with *Brucella*.

   b. Total IgG1 - Old infected mice produced markedly higher amount of IgG1 only on week 3 in comparison to uninfected mice. Young infected mice produced significantly higher amount of IgG1 on week 3 and week 5 as compared to control mice (Fig. 21B).

   c. Total IgG2a - Only the young infected mice produced significantly higher amount of IgG2a antibodies in comparison to uninfected young mice week 3 and week 5 post-infection. These young infected mice also produced significantly higher amount of total IgG2a in comparison to old infected mice on week 3 and week 5 (Fig. 21C).

6) **Determination of total IgG, IgG1 and IgG2a against Cu-Zn Superoxide dismutase (SOD)**

   a. Total IgG - Old mice infected with *B. abortus* produced significantly higher levels of IgG compared to uninfected mice on week 1 and week 5 whereas
young mice infected with *B. abortus* produced significantly higher levels of IgG at all
time points in comparison to young uninfected mice (Fig. 22A). In case of SOD, old
infected mice produced significantly higher levels of total IgG compared to young
infected mice on week 5 post-infection.

b. Total IgG1- Neither old nor young infected mice produced markedly
higher levels of IgG1 over uninfected mice (Fig. 22B).

c. Total IgG2a - Only the old infected mice produced significantly higher
levels of IgG2a antibodies in comparison to uninfected young mice on week 5 post-
infecction. The old infected mice also produced significantly higher levels of total
IgG2a in comparison to young infected mice on week 5 after infection (Fig. 22C).
Fig. 10: PCR amplification of \( bfr \) gene. Lane M. 1 kb plus DNA ladder; Lane 1 and Lane 2- PCR amplified \( bfr \) gene.

Fig. 11: Schematic representation of pRSET vector.
Fig. 12: Restriction digestion of pRSET- BFR construct
Lane M- 1 kb plus DNA marker, Lane 1, 2 and 3 –
Restriction digestion of pRSET vector containing the
bacterioferritin gene, Lane 4 and 5 – Restriction digestion
of pRSET vector only (without insert).
pRSET-BFR was transformed into *E. coli* BL21 cells and cell lysate was incubated with lysis buffer. It was mixed with Ni-NTA resin and loaded onto polypropylene column. The recombinant protein with polyhistidine tag was eluted and various eluted fractions were analyzed by SDS-PAGE for the presence of recombinant BFR protein. Lane M - Molecular weight marker; Lane L - Cell lysate. Lane 1 to Lane 8 - Serially eluted fractions 1 through 8 of bacterioferritin protein.
Fig. 14: Western Blotting analysis of eluted fractions of bacterioferritin protein using Histidine tag antibodies, Lane M - Molecular weight marker; Lane E1 to E3- Serially eluted fractions 1, 2 and 3 of bacterioferritin protein.
Fig. 15: SDS-PAGE analysis of bacterioferritin protein after dialysis.

All the serially eluted fractions containing the desired protein were pooled together and dialyzed using a dialysis cassette against phosphate buffered saline (PBS) for 72-96 hours and then the dialyzed protein was analyzed by SDS-PAGE. Lane M - Molecular weight marker; Lane 1 and Lane 2 – Dialyzed bacterioferritin protein.
Fig. 16: PCR amplification of \textit{sodC} gene.

Lane M- 1 kb plus DNA ladder, Lane 1 and Lane 2

PCR amplified superoxide dismutase gene.
Fig. 17: Restriction digestion of pRSET-SOD construct: Lane M – 1 kb plus DNA ladder. Lane 1, 2, 3 and 4 – Restriction digestion of pRSET vector only (without insert). Lane 5 – Restriction digestion of pRSET containing the sodC gene.
Fig. 18: SDS-PAGE analysis of the purified SOD protein.

pRSET-SOD plasmid was transformed into *E. coli* BL21 cells and cell lysate was incubated with lysis buffer. It was mixed with Ni-NTA resin and loaded onto polypropylene column. The recombinant protein with polyhistidine tag was eluted and various eluted fractions were analyzed by SDS-PAGE for the presence of recombinant SOD protein. Lane M – Molecular weight marker, Lane 1, 2, 3, 4 and 5 - Eluted fractions of SOD protein.
Fig. 19: SDS-PAGE analysis of SOD recombinant protein after dialysis. All the serially eluted fractions containing the desired protein were pooled together and dialyzed using a dialysis cassette against Phosphate buffered saline (PBS) for 72-96 hours and then the dialyzed protein was analyzed by SDS-PAGE. Lane 1 and 2 - Dialyzed SOD protein; Lane M- Molecular weight marker.
Fig. 20: Western Blotting analysis of eluted fractions of SOD protein using anti-Histidine antibodies. Lane M- Molecular weight marker, Lane N- Negative Control (uninduced BL21 cells-with pRSET vector only), Lane E1 to E3- Eluted fractions 1, 2 and 3 containing SOD protein.
Fig. 21 A, B and C- Total serum A) IgG B) IgG1 and C) IgG2a vs. the bacterioferritin protein after *B. abortus* infection in old vs. young DBA/2 mice. a, $p \leq 0.05$; b, $p \leq 0.01$; c, $p \leq 0.001$
Fig. 22 A, B and C- Total serum A) IgG, B) IgG1, and C) IgG2a vs. the SOD protein after infection with *B. abortus* in old vs. young DBA/2 mice; a. *p* ≤ 0.05; b, *p* ≤ 0.01; c, *p* ≤ 0.001
7) **Analysis of post-infection immune response in old vs. young BALB/c mice using RT-PCR and cytokine antibody array**

**RT-PCR:**

**Th1** - Various components of Th1 immunity were analyzed. Aged mice and humans exhibit a shift from Th1-dominant to a mixed or Th2-dominant response. So, it is important to characterize the Th1 immunity in aged mice.

IFN-γ is a key Th1 cytokine responsible for immunity against intracellular pathogens. According to our data young mice produced IFN-γ as early as day 1 pi, whereas old mice started producing IFN-γ by day 7 (Table 2). We analyzed the expression of IL-12R, which is present on Th1 cells was analyzed. This receptor consists of two subunits IL-12Rβ1 and IL-12Rβ2. IL-12Rβ1 is a constitutively expressed subunit and IL-12Rβ2 is induced. Importantly, IL-12Rβ2 expression correlates with a cell's ability to respond to IL-12. According to our data, both old and young mice expressed this receptor subunit on day 7 pi (Table 2). Functional IL-12 is composed of two subunits- p35 and p40. IL-12 p40 is a common subunit between IL-12 and IL-23, so p35 is unique to IL-12. During an initial encounter with an antigen, naive T cells respond to IL-12 by differentiating into a population of Th1-cells capable of producing large amounts of IFN-γ. Both old and young mice started producing IL-12 p35 just 1 day pi and was not expressed in control mice. In addition to these cytokines, T-bet, transcription factor for Th1 was also analyzed and it was found that old mice expressed T-bet 1 day pi. IL-27 is a cytokine of IL-12 family.
which promotes Th1-type immunity. The interesting finding in our experiment was that, IL-27 was expressed by old uninfected mice but there was no IFN-γ production in these mice. IL-27 was also expressed by both old and young infected mice. IL-27 does not drive the differentiation process of CD4 T cells toward an IFN-γ secreting phenotype. It actually acts on naïve CD4 T cells by inducing IL-12 responsiveness by means of T-bet induction, so in absence of IL-12, it cannot bias the T cell response towards a Th1 phenotype. Th1 chemokines such as CXCL9 and CXCL10 were also included in the analysis. These chemokines were produced by the young mice even in the absence of infection whereas old mice started producing the chemokines just 1 day pi (Table 3).

**Th2** - Th2-type response was also studied in this experiment. IL-4, the signature Th2 cytokine, was present in old and young mice 14 day pi (Table 4). GATA-3, Th2 transcription factor, was present in the old and young mice 1 day pi but in spite of presence of GATA-3, IL-4 was not produced.

**Th17** - IL-6 and TGF-beta are the principal cytokines responsible for the development of IL-17 producing T-helper cells (Th17). It was clear from the data (Table 5) that IL-6 was induced upon infection with *Brucella* in both young and old mice very early (1 day pi), whereas TGF-β was present constitutively in both age groups. IL-23, another important cytokine of IL-12 family, is responsible for the differentiation and survival of Th17 cells, was produced by all old and young mice 7 day pi. IL-17A is the functional cytokine of the IL-17 family. In addition to IL-17A, IL-17F was also produced by all old and young mice 14-day pi. The most interesting observation here is IL-17A, which is the functional form of IL-17, was produced only...
by old mice; whereas both age groups produce IL-17F. IL-17A and IL-17F are secreted by Th17 cells. We also examined the expression of IL-23R that is expressed on Th17 cells which enable them to be responsive to IL-23. According to our control mice data, old mice expressed IL-23R even in absence of infection but IL-17 was not produced; the reason may be the lack of expression of IL-6 in uninfected mice. ROR-γt, transcription factor for Th17 cells, was also expressed early only by old mice as early as 7-day pi and produced by all old and young mice by day 14 pi (Table 5).

**Cytokine antibody array:**

**Th1**- It is also evident from the assay that old mice produced more IFN-γ than young mice post-infection. Functional IL-12p70 was higher in young mice whereas old mice produced more IL-12 p40 subunit. This may indicate that either old mice have more free IL-12 p40 subunit or it is used in the synthesis of another IL-12p40 containing cytokine i.e. IL-23 (Fig. 23). We also analyzed MIG, a Th1 chemokine also known as CXCL9 and found that old mice produce higher level of MIG and RANTES than young mice (Fig. 23).

**Th2**- IL-4, key Th2 cytokine and IL-10 were produced by both old and young mice almost at the same level (Fig. 24).

**Th17**- It is evident from the graph that old mice expressed more IL-17 than young mice. The level of IL-6 was also higher in old mice. TGF-β and IL-23 were not included on the array. Other cytokine produced by Th17 cells like KC, MIP-2 and MIP-3α were also increased in old mice (Fig. 25).
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Table 2: Analysis of Th1 cytokines and transcription factors by RT-PCR

n = Number of mice in each group; 0, 1, 2, 3, 4 indicates the number of mice found positive for that particular cytokine.
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Table 3: Analysis of Th1 chemokines by RT-PCR

n = Number of mice in each group; 0, 1, 2, 3, 4 indicates the number of mice found positive for that particular cytokine.

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Table 4: Analysis of Th2 cytokines by RT-PCR

n = Number of mice in each group; 0, 1, 2, 3, 4 indicates the number of mice found positive for that particular cytokine.
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Table 5: Analysis of Th17 cytokines and transcription factors by RT-PCR

n = Number of mice in each group; 0, 1, 2, 3, 4 indicates the number of mice found positive for that particular cytokine.
Fig. 23: Analysis of Th1 cytokines and chemokines identified using cytokine antibody array.
Fig. 24: Analysis of Th2 cytokines identified using cytokine antibody array.
Fig. 25: Analysis of Th17 cytokines and chemokines identified using cytokine antibody array.
CHAPTER 5

DISCUSSION

*Brucella* is a Gram-negative facultative intracellular bacterium which has developed a unique way to survive and multiply inside the phagocytic cells of the host. *Brucella* infection results in induction of Th1 response necessary for the clearance of the organism. In the current study, we demonstrated that old DBA/2 mice were able to clear infection due to *B. abortus* strain 2308 as efficiently as young mice. It is evident from the data (Fig. 3) that old mice have higher CFU/spleen at week 1, equivalent at week 3 and significantly lower CFU/spleen at week 5 than young mice. There are very few examples regarding the capacity of old mice to handle infection more efficiently than young mice. Cooper et al (1995) demonstrated that older mice have enhanced resistance against mycobacterial infection in comparison to young mice, but this transient resistance was evident only in the first three weeks of infection. By day 40, young mice showed a better rate of clearance. Subsequently, it was shown that the transient resistance of older mice to mycobacterial infection was mediated by resident CD8 T cells in the lung capable of secreting IFN-γ (Turner et al, 2002). This transient resistance was lost in aged CD8-gene disrupted mice. But the capacity of aged mice to show an early resistance was not evident when the mice were infected through an intravenous route indicating the presence of a specific mechanism within the lung responsible for efficient clearance of the infection (Vesosky et al, 2005). Ehrchen et al (2004) demonstrated that senescent BALB/c mice infected with *Leishmania major* showed ulceration of footpads after 7 weeks of infection in the same manner as young mice but the ulceration healed in 60% in senescent mice after 10 weeks of infection. The ability of
senescent mice to exhibit a protective immune response towards *L. major* infection was associated with spontaneous release of IL-12 by macrophages from aged mice but not from young mice. CD4 lymph node cells from infected senescent mice also showed a release of significantly higher amount of IFN-γ when restimulated with soluble leishmanial antigen. But, it is evident from our data that old mice were able to clear infection more efficiently than young mice at week 5 post-infection. One of the possible mechanisms by which aged mice efficiently clear the *Brucella* organisms may be with the host immune cells, particularly macrophages might not be able to support the growth of intracellular *Brucella*. Muller et al (2008) reported that younger BALB/c mice showed severe lesion pathology and higher parasite burdens in comparison to aged mice in response to *Leishmania* infection. They concluded that the profile of helper T cell response is not the reason for the young mice to demonstrate this kind of outcome but the macrophages from older mice were less favorable for parasite growth than those of young mice due to limited nutrient availability. The macrophages from young mice have increased expression of arginase that catalyzes the conversion of L-arginine into urea and ornithine, the latter being used by the parasite for growth; whereas macrophages from older mice have reduced arginase activity. Macrophages contain two enzymes - nitric acid synthase 2 (NOS2) and arginase; both the enzymes compete for a common substrate, L-arginine and both are regulated by type 1 and type 2 cytokines respectively. The type 1 cytokine IFN-γ induces conversion of arginine into nitric oxide (NO) which is instrumental in parasite killing whereas type 2 cytokine IL-4 induces arginase and in turn helps in parasite growth. It is reported that the capacity to generate NO by
macrophages from older mice remains unaltered but at the same time, their arginase activity decreases in comparison to young macrophages. So, older macrophages possess the capacity to kill the parasites with the help of NO and the reduced arginase activity results in unfavorable environment for parasite growth. It can be deduced from our data that the CFU/spleen of old mice were significantly higher than that of young mice at week 1 post-infection but the aged mice cleared the infection more efficiently than young mice on week 5 post-infection. These results negate the possibility that macrophages from older mice can't support the growth of *Brucella* intracellularly. The outcome of *Brucella* infection in aging model can be attributed to the unique intracellular replication and survival strategies adopted by this organism (Letesson et al, 2002; Celli, 2006).

In the second study, the capacity of strain RB51-SOD vaccination to provide protection against *B. abortus* 2308 infection were compared in old and young DBA/2 and BALB/c strain of mice. BALB/c and DBA/2 strains of mice demonstrate the Th2-biased phenotype (so considered as *Brucella* susceptible) whereas C57BL/6 are considered as *Brucella* resistant owing to their Th1-orientation (High, 2006). BALB/c mice serve as an established model of aging and are being used widely in aging studies (Bates et al, 2008; Muller et al, 2008; Ehrchen et al, 2004; Vesosky et al, 2006; Maletto et al, 2005). It can be inferred from the data that strain RB51-SOD vaccination provided significant protection against strain 2308 infection in old BALB/c, young BALB/c and young DBA/2 mice but not in old DBA/2 mice (Fig 8A and Fig. 8B). It is also clear from the data that there is a strain difference in terms of organism burden in unvaccinated mice from both age groups. Both old and young
DBA/2 unvaccinated mice have an organism burden significantly lower than their counterparts of BALB/c strain (Fig. 7A). It means that DBA/2 mice have the natural resistance to *Brucella* infection in comparison to BALB/c mice and this ability is not lost with age. This result is consistent with the findings of Howard et al (1980) who also reported resistance of DBA/2 mice and susceptibility of BALB/c mice to *L. major* infection. The data also suggests that strain RB51-SOD vaccination provides a significant level of protection against strain 2308 challenge in young mice of both the genotypes (Fig. 8A). However, the vaccination provides significant protection in old BALB/c mice but not in old DBA/2 mice (Fig 8B). One reason may be the ability of naïve DBA/2 mice to clear *Brucella* infection more efficiently than naïve BALB/c mice thus making the gap narrower between naïve and vaccinated DBA/2 mice but not in BALB/c mice. It should be noted that DBA/2 mice carry the resistance allele of the antimicrobial natural resistance gene, Nramp1 whereas BALB/c carry the susceptible allele (Malo et al, 1994). This can be an explanation of the ability of DBA/2 mice to handle *Brucella* infection more efficiently than BALB/c mice. The mouse chromosome 1 locus Bcg controls the capacity of tissue macrophages to restrict the replication of intracellular pathogens like *Mycobacterium* sp, *Salmonella* and *Leishmania* (Potter et al, 1983; Mazzolla et al, 2002).

In order to understand the mechanism of defense vs. *B. abortus* infection in older mice, total IgG, IgG1 and IgG2a level was measured to two immunodominant antigens of *Brucella*, Cu-Zn Superoxide dismutase (SOD) and Bacterioferritin (BFR). IgG1 and IgG2a antibody isotypes are indicators of Th2 and Th1 response respectively. It can be deduced from the data that older mice can elicit as strong
antibody response to *Brucella* infection as young adult mice. But, there is a notable difference in immune response of older mice to the two antigens of *Brucella*. The older infected mice produced significantly higher amount of IgG2a antibodies, indicator of Th1 immunity, against SOD in comparison to young infected mice (Fig. 22C) but the response to BFR was exactly opposite. In this case, young adult mice produced significantly higher IgG2a response in comparison to young mice on week 3 and week 5 post-infection (Fig 21C). These data indicate that older mice have the ability to mount Th1 response against *Brucella* infection. The diversity of T cell receptor is drastically reduced due to aging (Naylor et al., 2005), this can be a reason for the difference in magnitude of immune response of older mice to SOD or BFR.

Subsequently, various components of Th1, Th2 and Th17 immune response were characterized using Reverse transcription PCR (RT-PCR) and cytokine antibody array. The data clearly suggests that old infected mice produced higher levels of IL-17 and IFN-γ in comparison to young infected mice (Fig. 23 and 25). IL-17 and IFN-γ are the key cytokines released from Th17 and Th1 cells respectively. CD4 effector T cells have been traditionally categorized into two subsets - Th1 and Th2. Th1 cells are highly effective in clearing intracellular pathogens and produce higher amount of IFN-γ. In contrast, Th2 cells are important in protection against extracellular parasites and produce IL-4, IL-5 and IL-13. In addition to Th1 and Th2 cells, Th17 is the distinct subset of T helper cells unrelated to either Th1 or Th2 cells. These immune cells produce cytokine such as IL-17 and IL-22 (Betteli et al, 2007). Naïve T cells activated in the presence of TGF-β and IL-6 differentiate into Th17 subset. The main function of IL-6 is to upregulate the expression of IL-23 receptor (IL-23R) to further
the development of these cells. Th17 cells expressing IL-23R bind to IL-23. IL-23 is a key cytokine inducing expansion of IL-17 producing CD4 T cells. It is evident from our data that old mice produced elevated level of IL-6 (Fig. 25). Increased IL-6 level has been shown to be associated with aging (Dayhoff et al, 2008). Increased IL-6 level is favorable for the development of Th17 cells in old mice. In addition to these cytokines, Th17 cells also regulate the immune response through production of chemokines like KC and MIP-3α, these chemokines play an important role in inflammation by increasing the mobilization of neutrophils (Kastelein et al, 2007). Old mice produced higher level of KC in comparison to young infected mice (Fig. 25). We also performed differential counts on blood samples collected from these old and young mice and found that old infected mice have significantly higher count of neutrophils in comparison to young infected mice (data not shown). This can be the result of downstream effect of increased IL-17 expression in old infected mice. IL-17 is not only involved in autoimmune diseases but also plays a role in host defense. IL-17 has been shown to improve host defense in case of Klebsiella pneumoniae, Mycoplasma pneumoniae, Bordatella pertussis and M. tuberculosis.

Till recently, there are few reports which link accentuated Th17 response with aging. According to the first report, vascular expression of IL-17 was found to be increased due to aging (Csiszar et al, 2003). In a recent study, Huang et al (2008) reported that splenic CD4 T cells from old C57BL6 mice generated 20-fold more IL-17 and 3-fold more IL-6 than that of young mice when stimulated with CD3 plus T cell receptor antibodies.
According to the results in this thesis, older mice produced higher levels of IFN-γ than young mice against *Brucella* infection (Fig. 23). IFN-γ is a key cytokine released from Th1 cells and it plays a pivotal role in *Brucella* immune response. IL-12 promotes the differentiation of naïve CD4 T cells to IFN-γ-secreting CD4 T cells through IL-12 receptor; this receptor consists of two components– IL-12Rβ1 and IL-12Rβ2. Functional IL-12 i.e. IL-12 p70 is made up of two subunits- IL-12p35 and IL-12p40. Coexpression of both subunits of IL-12 in one cell is required for the generation of biologically active IL-12 (Trinchieri et al, 2003). In the late 1990s, another cytokine was discovered that shares its p40 subunit with IL-12. The cytokine known as IL-23 has two subunits- p40 and a unique p19 subunit (Oppmann et al, 2000). Later on, it was found that IL-23 promotes the development of Th17 cells.

The presence of p40-dependent and IL-12p70-independent mechanism of resistance was reported in a *Mycobacterium* infection model (Cooper et al, 2002). But it was not certain whether the p40-dependent resistance to *Mycobacterium* was indeed mediated by IL-23. Subsequent studies (Khader et al, 2005; Wozniak et al, 2006) proved that IL-23 mediates protection to *Mycobacterium* infection in the absence of IL-12p70. Similar findings were also observed in case of toxoplasmosis (Lieberman et al, 2004) where IL-23 can provide a limited mechanism of resistance to the infection in absence of IL-12. Even in the murine model of fungal infection with *Cryptococcus neoformans* (Kleinschek et al, 2006), IL-23 mediates protection against chronic fungal infection. IL-23 has the capacity to generate IFN-γ-producing CD4 T cells (Khader et al, 2005). IL-23 is also involved in stabilizing and maintaining IL-17 producing CD4 T cells (Weaver et al, 2006). The dual role of IL-23 in driving both
IFN-γ and IL-17 responses was also reported in a Helicobacter hepaticus-induced T-cell-dependent colitis model (Kullberg et al, 2006).

Our preliminary results also suggest about increased IFN-γ response by older mice in a setting of increased p40 and decreased p70 expression compared to young mice (Fig. 23). The results from our collaborators at Wake Forest University also showed an increased secretion of IL-23 by dendritic cells from old mice after Brucella infection (data not shown). This increased p40 expressed by the older infected mice may be utilized in synthesizing IL-23. There is possibility of the involvement of IL-23 in generating Th1 response in older mice resulting in increased IFN-γ production. In another study on aged mouse model of respiratory syncitial virus (RSV) infection, it was reported that the older BALB/c have diminished RSV-specific CD8 CTL response and reduced IFN-γ response in comparison to young mice (Zhang et al, 2001) which may account for the increased mortality and morbidity in elderly people due to RSV infection.

In addition to analysis of different cytokine by cytokine antibody array, RT-PCR was also performed to further understand the role of various cytokines. There are some cytokines and transcription factors which are not included on the array like TGF-β, IL-23, T-bet, ROR-γt, GATA-3 etc. T-bet is the most important transcription factor for Th1 development (Szabo et al, 2000). Similarly, ROR-γt and GATA-3 play a vital role in the development of Th17 (Ivanov et al, 2006) and Th2 cells (Zheng and Flavell, 1997). Our results indicate that old mice show an early expression of Th17 transcription factor, ROR-γt and IL-17 (Table 5). In addition, old mice have the capacity to express IL-23p19 same as young mice. It is also clear from the data that
old mice have the ability to express Th1 transcription factor, T-bet and IL-12Rβ2 (Table 2) which explains the ability of old mice to produce IFN-γ.

This study has important ramifications. *Brucella* has been used as a Th1-inducing carrier system to express heterologous antigens (Vemulapalli et al, 2000). We have demonstrated that old mice possess the capacity of a better resistance to *Brucella* infection. Further investigation into the mechanism of resistance in older mice will facilitate the understanding of the components of immunity which are intact even in advanced age. These pathways can then be exploited to design new strategies to improve host defense in the elderly. The inherent ability of *Brucella* to invoke Th1 immunity and act as a host for heterologous antigen expression makes it a promising vehicle for delivery of protective antigens to aged people and warrants further investigation.
CHAPTER 6

SUMMARY AND CONCLUSIONS

Aging results in waning of immunity to intracellular pathogens leading to increased mortality and morbidity. There is plethora of data suggesting decreased resistance of aged individuals to infections due to intracellular pathogens like *Mycobacterium, Salmonella, Listeria, Toxoplasma, Influenza virus*, but there is not enough data on effect of aging on immune response against *Brucella*. There is an increased interest in research on *Brucella* because of the classification of this organism as a potential bioterrorism agent. Therefore, the present study was performed to evaluate the effect of aging on immune response against *Brucella* infection.

Based on the results, I am proposing a model of potential mechanism of *Brucella* adaptive immunity in aged vs. young mice (Fig. 26). Our preliminary results demonstrate that old mice have the capacity to generate a brisk Th1 as well as Th17 response (Fig. 23, 25) due to *Brucella* infection. This was validated by performing cytokine RT-PCR and cytokine array for different components of adaptive immunity such as Th1, Th2 and Th17. It is also evident that the shifting of aging immune system towards Th2 does not apply to the *Brucella* model of infection. High level of IL-6 in old mice (Fig. 25) can be a contributing factor towards generation of more Th17 cells which in turn secretes high level of IL-17 (Fig.25). IL-17 is known to produce neutrophilia due to its activity that is also observed in old mice. Old mice were found to contain significantly increased number of neutrophils in comparison to young mice on day 7 (43 ± 4.3 vs. 14 ± 5.7) and day 14 (60 ± 6.0 vs. 33 ± 5.2) post-infection.
Fig. 26: Proposed mechanism of *Brucella* adaptive immunity in aged vs. young mice
We also analyzed some of the chemokines produced by Th17 as well as Th1 cells. Old mice produce higher level of Th1 chemokines (Fig. 23), which can attract IFN-γ-producing T cells to the site of infection. Neutrophils are known to be the major producers of these chemokines. Even old mice have capacity to produce higher level of IFN-γ due to Brucella infection (Fig. 23), which is indicative of brisk Th1 response. The capacity of old mice to produce Th1 cells can be attributed to their ability to express T-bet as well as IL-12Rβ2 (Table 2). Old mice produced increased expression of IL-12p40 but impaired IL-12p70 level in comparison to young mice (Fig. 23). There is possibility of p40-dependent and p70-independent generation of IFN-γ producing T cells in old mice due to Brucella infection. Therefore, it can be concluded from our study that -

1) Old mice clear Brucella more efficiently than young mice.

2) Older mice have the capacity to mount a vigorous Th17 as well as Th1 response against Brucella infection in comparison to young mice.

3) IL-23/IL-17 axis may play an important role in aging immune system.

But how the Th17 as well as the Th1 branches of immune system work together enabling old mice to clear Brucella better than young mice is a matter of future investigation in our laboratory. Exploring this mechanism will provide a better insight into developing more effective vaccine strategies to control infectious diseases in the aged population.
CHAPTER 7

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